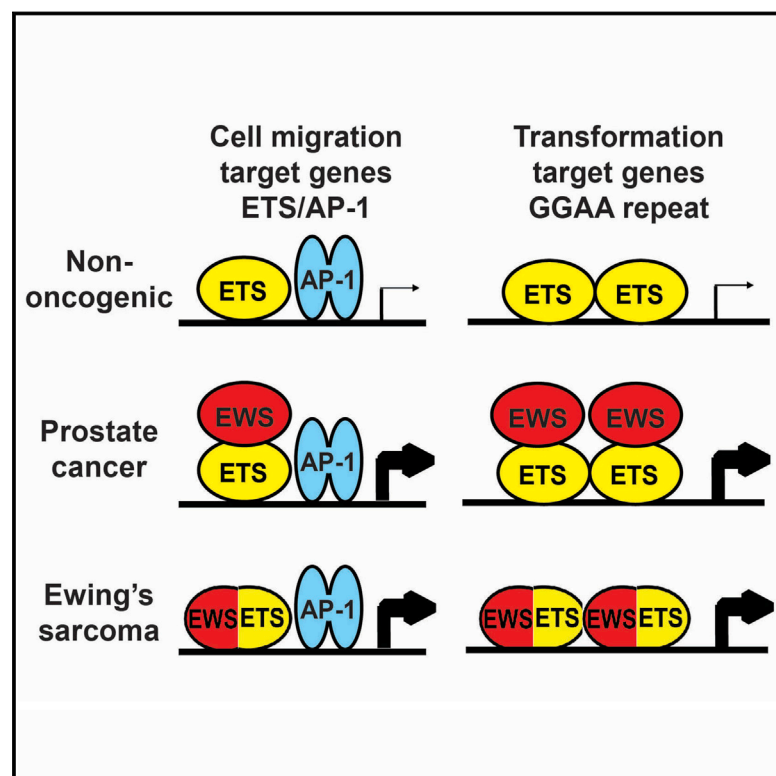


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An Interaction with Ewing's Sarcoma Breakpoint Protein EWS Defines a Specific Oncogenic Mechanism of ETS Factors Rearranged in Prostate Cancer

Graphical Abstract



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In Brief

A subset of ETS transcription factors is oncogenic in prostate. Kedge et al. show that oncogenic ETS, but not other ETS, interact with EWS, and this interaction is necessary for oncogenic functions. Because EWS is fused to ETS factors in Ewing's sarcoma, this finding links the mechanisms of these diseases.

Highlights

- EWS interacts specifically with ETS proteins that promote prostate cancer
- EWS is required for oncogenic ETS functions in prostate cells
- EWS acts as a co-activator for oncogenic ETS transcription factors
- Oncogenic ETS function through similar mechanisms in prostate and Ewing's sarcoma

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An Interaction with Ewing's Sarcoma Breakpoint Protein EWS Defines a Specific Oncogenic Mechanism of ETS Factors Rearranged in Prostate Cancer

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SUMMARY

More than 50% of prostate tumors have a chromosomal rearrangement resulting in aberrant expression of an oncogenic ETS family transcription factor. However, mechanisms that differentiate the function of oncogenic ETS factors expressed in prostate tumors from non-oncogenic ETS factors expressed in normal prostate are unknown. Here, we find that four oncogenic ETS (ERG, ETV1, ETV4, and ETV5), and no other ETS, interact with the Ewing's sarcoma breakpoint protein, EWS. This EWS interaction was necessary and sufficient for oncogenic ETS functions including gene activation, cell migration, clonogenic survival, and transformation. Significantly, the EWS interacting region of ERG has no homology with that of ETV1, ETV4, and ETV5. Therefore, this finding may explain how divergent ETS factors have a common oncogenic function. Strikingly, EWS is fused to various ETS factors by the chromosome translocations that cause Ewing's sarcoma. Therefore, these findings link oncogenic ETS function in both prostate cancer and Ewing's sarcoma.

INTRODUCTION

In 50%–70% of prostate tumors, a chromosomal rearrangement results in fusion of a transcriptionally active promoter and 5' UTR to the open reading frame of an ETS family transcription factor, resulting in aberrant expression in prostate epithelia (Tomlins et al., 2005, 2007). The most common fusion is *TMPRSS2:ERG*, occurring in approximately one-half of prostate tumors. *ETV1* and *ETV4* rearrangements occur in an additional 5%–10% of tumors. Expression of *ERG*, *ETV1*, and *ETV4* in prostate cells is oncogenic, particularly when coupled with a second mutation activating the PI3K/AKT or androgen receptor pathways (Aytes et al., 2013; Baena et al., 2013; Carver et al., 2009; King et al., 2009; Zong et al., 2009). Fusion transcripts involving other ETS

genes (*ETV5*, *FLI1*, *EHF*, *ELF2*, *ELK4*, and *ETV6*) have been identified at low frequency (<2%), but it is not clear if these are oncogenic or passenger mutations (Rickman et al., 2009; Robinson et al., 2015). Although significant progress has been made in understanding the oncogenic gene targets of ERG, ETV1, and ETV4, the molecular mechanisms used by these three ETS transcription factors to activate transcription of these target genes are largely unknown. Furthermore, although these rearrangements tend to be mutually exclusive (Svensson et al., 2011), it is not clear if *ERG*, *ETV1*, and *ETV4* utilize a common molecular mechanism to promote prostate cancer.

The human genome encodes 28 ETS factors, and these are extensively co-expressed, with at least 15 present in any individual cell type (Hollenhorst et al., 2004). ETS proteins share a highly conserved ETS DNA binding domain and can be divided based on similarities in this domain into subfamilies of no more than three members each (Hollenhorst et al., 2011b). Within a subfamily, amino acid homology extends across the entire protein, but between subfamilies, the only homology is in the ETS DNA binding domain. ETV1, ETV4 (PEA3), and ETV5 comprise the PEA3 subfamily, but ERG is in a distinct subfamily, and therefore, outside of the DNA binding domain, ERG has no amino acid sequence similarity with ETV1, ETV4, and ETV5. Instead, ERG is homologous with FLI1 and FEV, which have no clear roles in prostate cancer. These sequence comparisons make it difficult to predict a conserved functional mechanism through which ERG, ETV1, and ETV4 promote prostate cancer that would not also extend to the non-oncogenic ETS factors normally expressed in prostate cells.

Oncogenic ETS proteins are known to promote cell migration, invasion, and epithelial-mesenchymal transition (EMT) when overexpressed in prostate epithelial cells (Tomlins et al., 2008; Wu et al., 2013). By directly comparing overexpression of multiple ETS factors in RWPE1 immortalized-normal prostate epithelial cells, we previously demonstrated that only ERG, ETV1, ETV4, and ETV5, but not FLI1 or other ETS proteins, promote cell migration (Hollenhorst et al., 2011a), indicating that these four ETS proteins share a common biological function that is unique in the ETS family. These ETS proteins activated transcription of cell migration genes by binding *cis*-regulatory sequences

that have neighboring ETS and AP-1 transcription factor binding sites. However, this ETS/AP-1 binding is not specific to ERG, ETV1, ETV4, and ETV5, because ETS1 can also bind ETS/AP-1 sequences and activate transcription of cell migration genes in KRAS mutant cancer cells (Plotnik et al., 2014). Therefore the molecular mechanism behind the specific biological function of ERG, ETV1, ETV4, and ETV5 in prostate cells is unknown.

Prostate cancer is not the only malignancy caused by ETS gene rearrangements. Ewing's sarcoma is caused by chromosomal translocations involving one of five different ETS genes. Prostate cancer chromosomal rearrangements generally promote expression of full-length or N-terminally truncated ETS proteins (Clark et al., 2007). In contrast, the oncogenic product of an Ewing's sarcoma translocation is a fusion protein consisting of an N-terminal domain of the RNA binding protein EWS fused to a C-terminal region of an ETS protein (Delattre et al., 1992; Patel et al., 2012). In this fusion oncoprotein, the N terminus of EWS contributes a strong transcriptional activation domain, and the C terminus of the ETS protein contributes the ETS DNA binding domain, both of which are necessary for transformation of Ewing's sarcoma cells (May et al., 1993). Interestingly, the five ETS genes involved in Ewing's sarcoma fusions (*FLI1*, *ERG*, *FEV*, *ETV1*, and *ETV4*) partially overlap with those rearranged in prostate cancer. However, the most common Ewing's fusion is *EWS:FLI1* (85%), and *FLI1* has not been shown to drive prostate tumorigenesis and does not promote prostate cell migration (Hollenhorst et al., 2011a). Furthermore, there is no known connection between the molecular mechanism of prostate cancer ETS rearrangements and Ewing's sarcoma fusion proteins.

This study investigated the role of the wild-type EWS protein in the oncogenic mechanism of ETS genes rearranged in prostate cancer. EWS was found to interact specifically with the ETS proteins ERG, ETV1, ETV4, and ETV5. This interaction occurred both in cell lines and between purified proteins, indicating it is direct. Fusion of the EWS N terminus with any ETS protein promoted prostate cell migration, indicating that this interaction is sufficient for an oncogenic phenotype. Furthermore, using both a knockdown of EWS and a point mutation in ERG that fails to interact with EWS, we demonstrate that the EWS-ETS interaction is critical for oncogenic ETS proteins to activate gene expression and drive cell migration and transformation in prostate cells. Activation of gene expression via ETS and EWS occurred through both ETS/AP-1 and GGAA-repeat *cis*-regulatory sequences. The necessity of EWS was specific to cell lines that express oncogenic ETS proteins, indicating that therapeutic strategies to inhibit this interaction may result in few side effects. These data suggest that a protein-protein interaction with EWS defines the specific oncogenic function of the ETS family transcription factors that drive prostate tumorigenesis and sets the stage for future work to further define this interaction in vivo and in human tumors.

RESULTS

EWS Interacts Specifically with ETS Transcription Factors that Promote Prostate Cancer

The EWS protein is fused to ETS transcription factors in Ewing's sarcoma, but the oncogenic role for wild-type EWS is less clear.

To test if EWS naturally interacts (absent fusion) with any ETS protein, we immobilized 21 purified full-length ETS proteins, representing every ETS subfamily, on beads, and we used these for affinity purification of interacting partners from PC3 prostate cancer cell nuclear extract. An immunoblot indicated that four of these 21 ETS proteins strongly and specifically interact with EWS (Figure 1A). Surprisingly, these four ETS proteins (ERG, ETV1, ETV4, and ETV5) cannot be grouped by common sequence but are linked by a common biological function: the ability to promote prostate cancer. This indicates that an interaction with EWS could define a common molecular mechanism for prostate cancer ETS oncoproteins.

To test if this interaction occurs in cancer cells, two prostate cancer cell lines that overexpress an oncogenic ETS factor were examined. VCaP cells have a *TMPRSS2:ERG* fusion (Tomlins et al., 2005), and immunoprecipitation with an anti-ERG antibody enriched for EWS (Figure 1B). PC3 cells overexpress ETV4 (Hollenhorst et al., 2011c), and immunoprecipitation with an anti-ETV4 antibody also enriched for EWS (Figure 1C). Reverse co-immunoprecipitation with anti-EWS antibody enriched for ERG (Figure 1B) and ETV4 (Figure 1C). EWS also co-immunoprecipitated with ERG in an ERG-positive primary prostate tumor (Figure 1D). To further validate the in vivo interaction, both ERG and EWS genomic occupancy was mapped in VCaP cells by chromatin immunoprecipitation sequencing (ChIP-seq). ERG-bound regions showed significant overlap with those identified in previous studies (Chng et al., 2012; Yu et al., 2010) and were enriched for ETS binding sites and histone H3 acetylation (Figures S1A and S1B). EWS co-occupied 1,242 of these ERG-bound genomic regions (Figure 1E; Table S1), a greater than 3-fold enrichment over the random expectation ($p < 0.0001$). Together, these data indicate that oncogenic ETS proteins interact with EWS in prostate cancer cells.

To test if the interaction between oncogenic ETS proteins and EWS is direct, glutathione S-transferase (GST)-tagged full-length EWS was purified from bacteria (Figure 1F) and mixed with beads bound to purified full-length ETS proteins. ERG, ETV1, ETV4, and ETV5 interacted with purified EWS, while the close ERG homolog, FLI1, did not (Figure 1G). The interaction of ETV1 with EWS was dramatically weaker than ERG, ETV4, or ETV5, indicating that the ETV1-EWS interaction may require additional partner proteins or posttranslational modifications. Together, these data suggest a direct and specific interaction between EWS and oncogenic ETS proteins.

Fusion with EWS Allows Any ETS Factor to Function like the Four Oncogenic ETS

Overexpression of ERG, ETV1, ETV4, and ETV5, but no other ETS proteins, promotes migration of the normal prostate cell line RWPE1 (Hollenhorst et al., 2011a). Strikingly, even the close ERG homolog FLI1 lacks this function. However, in Ewing's sarcoma, both FLI1 and ERG are oncogenic when fused to the N terminus of EWS due to a chromosomal rearrangement. To test if fusion of EWS could impart an oncogenic function on ETS proteins expressed in prostate cells, we compared full-length wild-type versions of seven ETS proteins to the same proteins N-terminally fused to amino acids 1–264 of EWS. To avoid

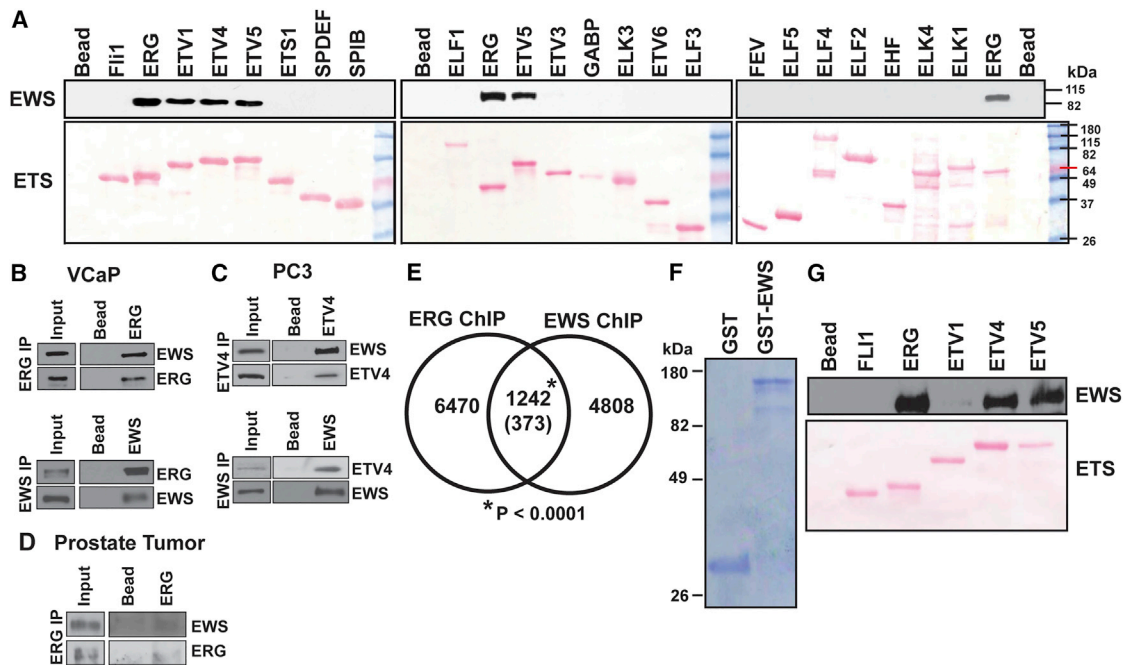


Figure 1. EWS Interacts Specifically with Oncogenic ETS Proteins

(A) Indicated purified His-ETS proteins were immobilized, incubated with PC3 nuclear extract, and washed. EWS binding was visualized by immunoblot (top) and ETS proteins by Ponceau stain (bottom).
 (B–D) Co-immunoprecipitation and reverse co-immunoprecipitation with indicated antibodies on extracts from VCaP (B) and PC3 (C) cell lines or a primary prostate tumor (D). Immunoglobulin G (IgG)-coated beads (beads) were used as control.
 (E) Overlap of bound regions identified by ChIP-seq in VCaP cells. Parenthesis indicates random prediction based on 125,000 possible binding sites.
 (F) SDS-PAGE stained with Coomassie brilliant blue showing GST and GST-EWS purified from bacteria.
 (G) Interaction of purified GST-EWS with purified, immobilized His-ETS proteins shown by anti-EWS immunoblot (top). His-ETS proteins were visualized by Ponceau stain (bottom).
 See also Figure S1 and Table S1.

super-physiological expression levels, the cytomegalovirus (CMV) promoter in the viral expression vector was replaced with the weaker *HNRNPA2B1* promoter, a promoter that drives ETS expression in some prostate tumor rearrangements (Tomlins et al., 2007). ERG expression from this vector was lower than ERG expression in VCaP cells or from a CMV vector (Figure S2A) but still caused a more than 3-fold increase in RWPE1 cell migration (Figure 2A). In contrast, expression of six non-oncogenic ETS proteins, including FLI1, did not significantly change cell migration (Figures 2A and S2B). Expression of the EWS N terminus by itself had no effect on cell migration, but every EWS-ETS fusion protein tested caused significant increases in cell migration. None of the EWS-ETS fusion proteins accumulated to appreciably higher levels in the cell than the ETS protein alone (Figure 2B). In Ewing's sarcoma fusions, the N terminus of ERG or FLI1 is removed and replaced by the N terminus of EWS. To test if this truncation of the ETS N terminus affected cell migration, we also tested EWS-NtermΔERG and EWS-NtermΔFLI1 constructs analogous to the fusion proteins found in Ewing's sarcoma. These EWS-NtermΔETS constructs drove cell migration to the same extent as EWS-ETS constructs (Figures 2A and 2B). Therefore, an obligate interaction with the EWS N terminus is sufficient to allow an ETS protein to act like an oncogenic ETS factor.

To test if this role of EWS was limited to cell migration, a second phenotype, clonogenic survival, was assayed (Figure 2C). ERG expression alone caused little increase in clonogenic survival of RWPE1 cells. Previous reports indicate that ERG and activated AKT are both required for tumor formation (Carver et al., 2009; King et al., 2009). Expression of myristoylated AKT increased AKT activation but alone had little effect on clonogenic survival. However, expression of both ERG and myristoylated AKT together resulted in a dramatic increase in clonogenic survival (Figure 2C). Similar to the cell migration phenotype, FLI1 expression with activated AKT caused significantly lower increases in clonogenic growth than ERG with activated AKT, but EWS fused to FLI1 was similar to ERG (Figure 2C). Both cell migration and clonogenic growth assays indicate that the EWS interaction is sufficient to allow non-oncogenic ETS proteins to function like ERG.

Identification of EWS-ETS Interaction Domains

To identify the region of ERG that interacts with EWS, various ERG truncations and deletions were purified and tested for the EWS interaction in PC3 nuclear extract (Figure 3A). A deletion of the C terminus of ERG after amino acid 391 resulted in a loss of EWS binding. However, a truncated ERG containing amino acids 275–455 retained EWS binding. Therefore, the

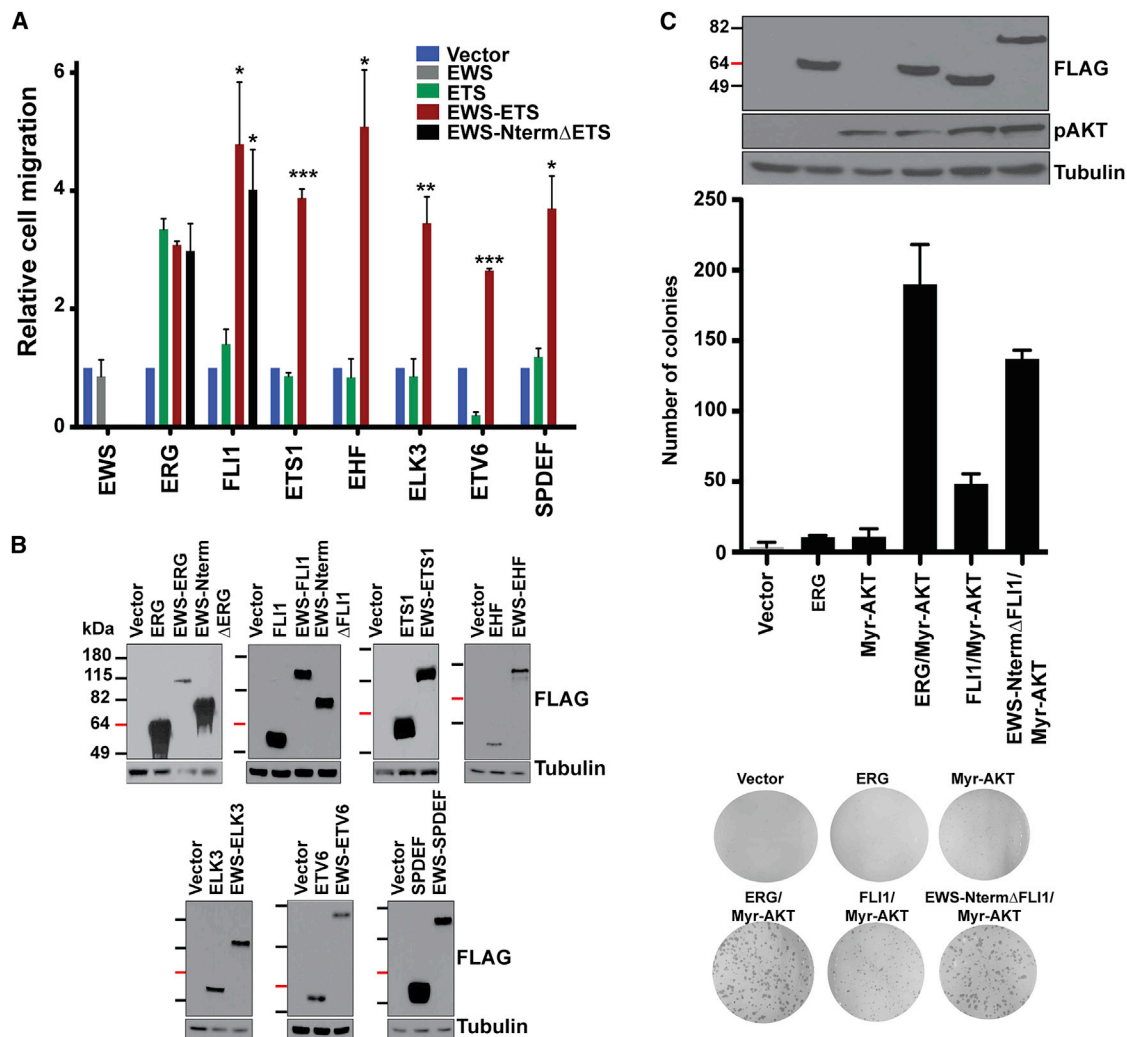


Figure 2. EWS-ETS Fusions Drive Prostate Cell Migration

(A) A transwell assay using RWPE1 cells stably expressing FLAG-tagged versions of the indicated ETS protein, EWS N terminus (1–264) alone, or EWS N terminus fused to an ETS. The fraction of migrated cells is shown relative to RWPE1 with empty vector as the mean and SEM of three replicates. p value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were obtained by t tests comparing EWS-ETS to corresponding ETS.

(B) Immunoblots indicate expression of indicated proteins with tubulin as a loading control.

(C) Immunoblot of indicated proteins (top). Clonogenic survival of RWPE1 cells expressing the indicated ETS and/or myristoylated AKT shown by mean and SEM of colony number (middle) or representative image (bottom).

See also Figure S2.

region of ERG, C-terminal to the ETS domain, between amino acids 391 and 455, was necessary for the EWS interaction. A deletion analysis of ETV5 identified a region necessary for EWS interaction that was N-terminal to the ETS domain spanning amino acids 357–368 (Figure 3B). This region of ETV5 has high homology to PEA3 subfamily proteins ETV1 and ETV4 but no homology to any region of ERG (Figures S3A and S3B). Similarly, the EWS interacting region of ERG had no homology with any region of ETV1, ETV4, or ETV5, indicating that ERG and PEA3 proteins interact with EWS through distinct interaction domains. The EWS interaction region of ERG has 75% identity with FLI1 (Figure S3A), which did not physically interact with EWS (Figure 1A). To identify the difference that allows ERG, but

not FLI1 to bind EWS, point mutations were created in ERG in amino acids that differ from FLI1 between 391 and 455. Two of these ERG point mutants were purified and tested for the interaction with EWS from PC3 cell nuclear extract (Figure 3C). One mutant, ERG P436A, eliminated the EWS interaction. Purified EWS protein was also used to show that ERG 1–391 and ERG P436A fail to directly interact with EWS (Figure 3D). To test if the P436A mutation disrupted the ERG-EWS interaction in cells, FLAG-ERG and FLAG-ERG P436A were expressed in RWPE1 cells and immunoprecipitated with anti-ERG antibody. Only ERG, and not ERG P436A, interacted with EWS (Figure 3E). Therefore ERG P436 is required for the EWS interaction both in cells and in vitro.

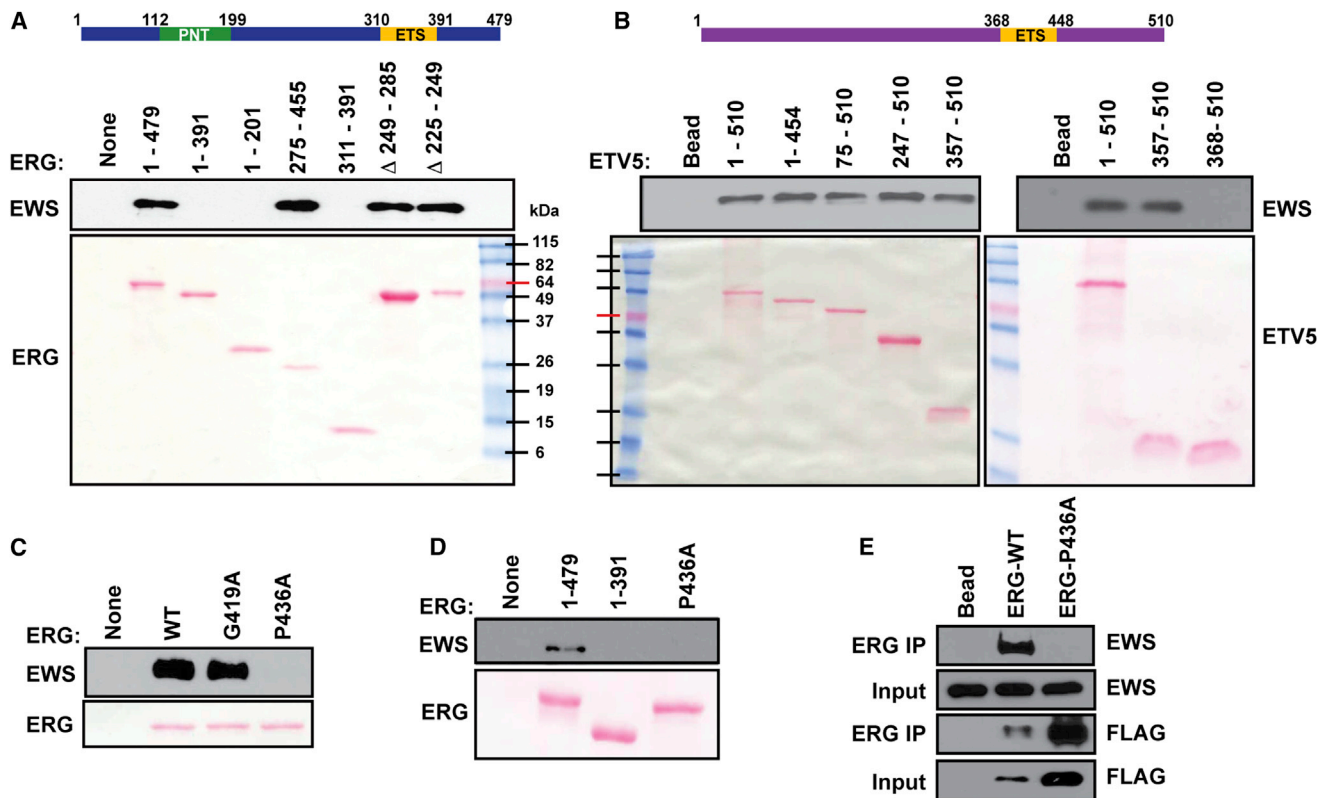


Figure 3. Identification of the EWS Interaction Domains

(A) A schematic of ERG protein showing the two known structured domains: pointed (PNT) and ETS DNA binding (ETS) (top). Indicated ERG constructs with a N-terminal His tag were immobilized to beads, mixed with PC3 nuclear extract, and washed. EWS binding was visualized by immunoblot (middle). Ponceau stain of the same blot shows the amount of His-ERG present (bottom).

(B) ETV5 has one known structured domain (ETS). The region of ETV5 that binds to EWS was mapped as in (A).

(C) EWS interaction with ERG as in (A), but with wild-type (WT) ERG or indicated ERG point mutants.

(D) Interaction of purified GST-EWS with indicated purified and immobilized His-ERG proteins. EWS binding is shown by immunoblot (top). His-ETS protein was visualized by Ponceau stain (bottom).

(E) Nuclear extracts prepared from RWPE1 cells stably expressing FLAG-ERG or FLAG-ERG P436A were immunoprecipitated (IP) with anti-ERG antibody (lanes 2 and 3) or bead-only control (lane 1) followed by immunoblotting for EWS or FLAG.

See also Figure S3.

The Interaction with EWS Is Necessary for Oncogenic ETS-Mediated Phenotypes, Including Tumorigenesis

Four oncogenic ETS proteins uniquely promote prostate cell migration (Hollenhorst et al., 2011a) and uniquely interact with EWS (Figure 1A). To test if the EWS interaction is required for the cell migration function, the ability of ERG and ERG P436A to promote migration of RWPE1 cells was compared. Despite being expressed at the same level, ERG promoted cell migration, and ERG-P436A inhibited cell migration (Figures 4A and S4A). This indicated not only that the EWS interaction was necessary for this ERG function but also that loss of the EWS interaction reversed ERG's function. A similar result was observed in the clonogenic growth assay, where the P436A mutation abrogated the ability of ERG to promote colony formation (Figure 4B). Neither ERG nor ERG P436A expression altered cell proliferation when plated at higher density (Figure S4B). To verify the necessity of EWS for an oncogenic ETS to promote cell migration, EWS was depleted from two prostate cancer cell lines, PC3 and

DU145, using two independent small hairpin RNAs (shRNAs) (Figure 4C). PC3 prostate cancer cells migrate due to overexpression of the oncogenic ETS ETV4 (Hollenhorst et al., 2011c), while DU145 prostate cancer cells migrate due to a KRAS gene rearrangement and do not express any of the oncogenic ETS (Selvaraj et al., 2014; Wang et al., 2011). EWS knockdown significantly decreased migration of PC3 cells, but not DU145 cells (Figures 4C and S4C). This indicates that EWS is required for cell migration in a cell line that expresses an oncogenic ETS, but not for cell migration in general. EWS knockdown had no effect on PC3 or DU145 cell proliferation (Figure S4D). To further confirm the specificity of EWS for oncogenic ETS-induced cell migration, we used the RWPE1 system. We have previously shown that either ERG or KRAS overexpression causes RWPE1 cells to migrate by activating a similar gene expression program (Hollenhorst et al., 2011a; Selvaraj et al., 2014). The necessity of EWS for this migration was compared by EWS knockdown in RWPE1-ERG and RWPE1-KRAS cells.

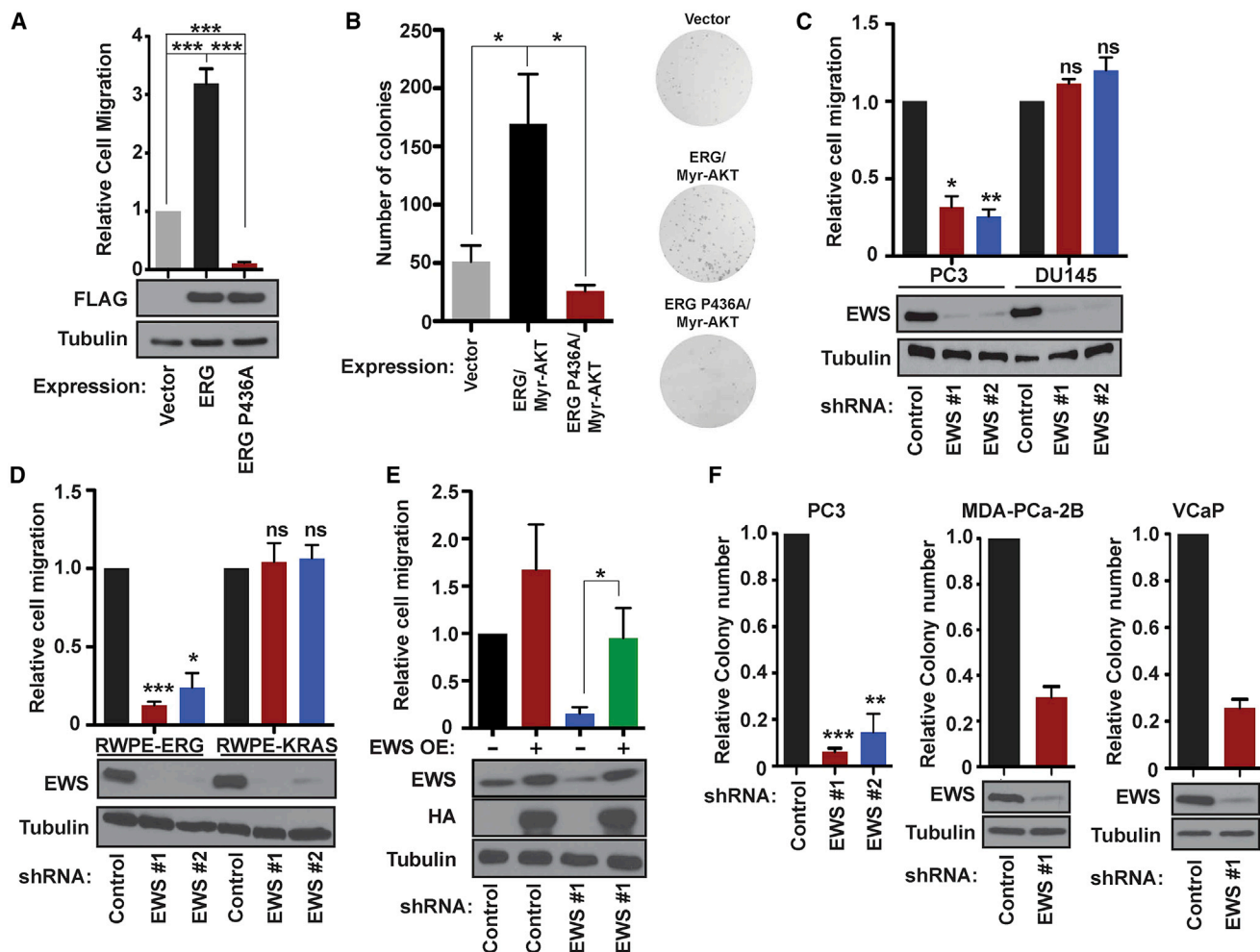


Figure 4. EWS Is Necessary for Oncogenic ETS-Mediated Phenotypes

(A) Transwell migration of RWPE1 cells expressing FLAG-ERG or FLAG-ERG P436A is shown relative to RWPE1 cells with empty vector as the mean and SEM of three biological replicates (top). Immunoblot of cells used in transwell assay (bottom).

(B) Mean and SEM of colony number and representative images of clonogenic growth of same lines used in (A); $n = 3$.

(C) Transwell migration of PC3 or DU145 prostate cancer cells stably expressing the indicated shRNA is shown relative to the negative control shRNA (targeting luciferase) as mean and SEM; $n = 3$ (top). Immunoblots of lines used in the transwell assay are shown (bottom).

(D) Migration as in (C) but using RWPE1 cells overexpressing ERG or KRAS as indicated.

(E) Migration of PC3 cells as (C) with or without EWS shRNA and 3xHA-EWS overexpression is indicated.

(F) Soft-agar growth from PC3 ($n = 3$), MDA-PCa-2B ($n = 2$), or VCaP ($n = 2$) cells with EWS knockdown as in (C) or as shown. Colonies present after 10 days relative to those expressing the control shRNA are shown as mean and SEM.

All p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns > 0.05) were obtained by t test (compared to control in the same cell line,) unless noted by a bracket. See also Figure S4.

EWS knockdown significantly reduced migration in ERG-overexpressing cells, but not the KRAS-overexpressing cells (Figures 4D and S4C). EWS knockdown had no effect on RWPE1-ERG or RWPE1-KRAS cell proliferation (Figure S4E). To show specificity, the EWS knockdown phenotype was rescued by EWS overexpression (Figures 4E and S4F). To extend these findings to another oncogenic ETS function, roles in anchorage-independent growth were tested. The oncogenic ETS protein ETV4 is required for anchorage-independent growth of PC3 prostate cancer cells (Hollenhorst et al., 2011c), and loss of EWS significantly inhibited growth of PC3 cells in soft agar (Figures 4F

and S4G). ETV1 and ERG are overexpressed in MDA-PCa-2B and VCaP cell lines, respectively, due to chromosomal rearrangements like those observed in patient tumors (Tomlins et al., 2007). EWS knockdown reduced soft-agar growth of both of these cell lines (Figures 4F and S4G). Together, these data show that the EWS interaction is responsible for common biological functions of oncogenic ETS proteins.

To directly test the role of the EWS-ERG interaction in tumor growth, we employed the established subcutaneous xenograft tumor growth model (Shaw et al., 2010). Since ERG accelerates tumor formation when expressed in prostate cells with activated

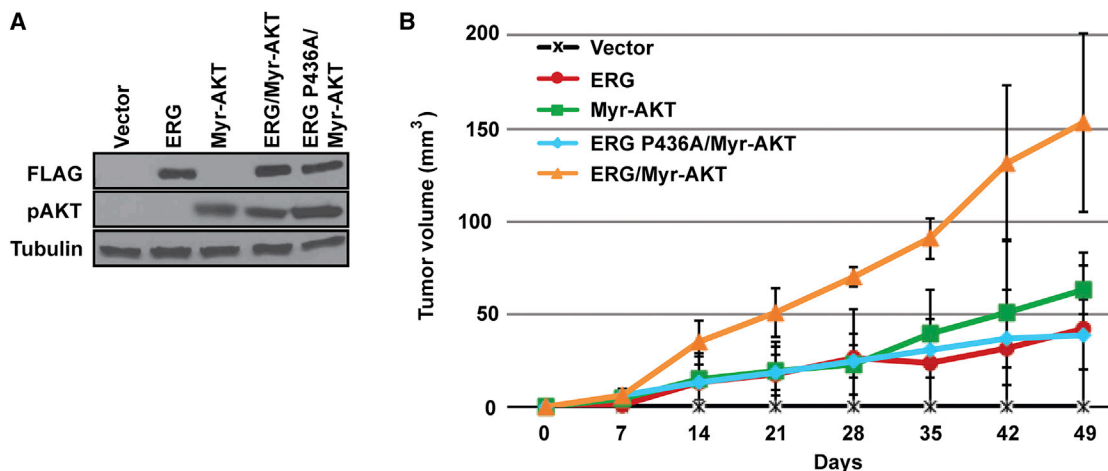


Figure 5. Tumor Growth Promoted by ERG Requires the EWS Interaction

(A) Immunoblots show expression of 3xFlag-ERG or phosphorylated AKT (pAKT) in RWPE1 cells expressing the indicated constructs.

(B) Cells from (A) were co-injected with cancer associated fibroblasts into the flanks of immunocompromised mice and tumor volume was measured by caliper at the indicated time points. Mean and SEM of six (ERG P436A/Myr-AKT) or twelve (all others) experiments are shown.

See also [Figure S5](#).

Akt signaling, we combined RWPE1 prostate cells stably expressing ERG, myristoylated AKT (myr-AKT), or both with cancer-associated fibroblasts subcutaneously into the flank of immunocompromised mice. ERG expression and AKT activation were confirmed by immunoblot ([Figure 5A](#)). An RWPE1 vector-only control formed no tumors, while RWPE1 cells expressing ERG or myr-AKT alone formed small masses that became stagnant after an initial growth phase between weeks 1 and 4 ([Figure 5B](#)). RWPE1 cells expressing both ERG and myr-AKT formed significantly larger tumors than cells expressing myr-AKT alone ($p = 0.043$, ANOVA, $n = 12$; [Figure 5B](#)), and these tumors continued to grow throughout the experiment, confirming that ERG is oncogenic. Importantly, the P436A point mutation of ERG abrogated this oncogenic function ([Figures 5B and S5A](#)). Although tumors expressing ERG and ERG-P436A had similar histology, there was a trend toward less proliferation in ERG-P436A tumors ([Figure S5B](#)). Together, these data indicate that the EWS interaction is required for the tumor-promoting function of ERG.

EWS Acts as a Co-activator for ERG

To activate gene expression, transcription factors generally bind to *cis*-regulatory elements and then recruit co-activator proteins that promote transcription. If EWS is recruited by ERG to target sites as a co-activator, we would expect the EWS binding mutant, ERG P436A, to bind target sites, but fail to recruit EWS resulting in loss of transcriptional activation. To test this hypothesis, ERG and ERG P436A were expressed in RWPE1 cells and genomic occupancy of ERG and EWS was assayed by ChIP. Six ERG target sites previously identified by ChIP-seq in this cell line ([Hollenhorst et al., 2011a](#)) showed very similar enrichment for both ERG and ERG P436A ([Figure 6A](#)), but EWS occupancy of the same genomic sites was significantly diminished by the ERG P436A mutant ([Figure 6B](#)). To test this if this pattern extended genome-wide, we first per-

formed ERG ChIP-seq in RWPE1-ERG cells to identify 1,901 ERG-bound regions. We then did independent ERG and EWS ChIP-seq in both ERG wild-type, and ERG-P436A expressing RWPE1 cells. In ERG-P436A-expressing cells, ERG enrichment actually increased compared to wild-type ERG, but EWS enrichment decreased ([Figures 6C, S6A, and S6B](#)). Peak calling indicated that a subset of ERG binding sites were occupied by EWS ([Figure S6A](#)). However, most of the “ERG alone” binding sites displayed EWS enrichment compared to neighboring chromatin, and this EWS enrichment also decreased in the ERG-P436A mutant ([Figure S6A](#)). It is possible that many of these sites are false negatives for EWS peak calling due to the difficulty of ChIP when a factor does not directly bind DNA. Together, these data indicate that the P436A mutation in ERG does not disrupt ERG chromatin occupancy but results in decreased EWS recruitment.

To test if loss of EWS from chromatin lowered gene expression, levels of two ERG target genes, *ARHGAP29* and *PIK3AP1*, was compared by qRT-PCR. Both were significantly lower when ERG P436A was expressed compared to ERG ([Figure 6D](#)). To extend this comparison genome-wide, we profiled in triplicate the transcriptome of RWPE1 cells expressing vector only, ERG, ERG-P436A, or ERG with an shRNA knockdown of EWS. 680 genes were significantly activated in the ERG-expressing cells compared to vector-only (2-fold or higher and adjusted p value < 0.05). ERG-activated genes were highly enriched for known ERG-regulated processes such as cellular migration and cellular adhesion and were enriched for neighboring ERG ChIP-seq binding sites ([Figures S6C and S6D](#)). ERG-activated transcripts were present at significantly lower levels in cells expressing ERG P436A or in ERG-expressing cells lacking EWS ([Figure 6E](#); [Table S2](#)) than in cells expressing wild-type ERG alone. In fact, 86% of ERG-induced genes showed lower expression in ERG-P436A and 87% were lower when ERG was expressed with EWS knockdown ([Figure S6E](#)). Together, these

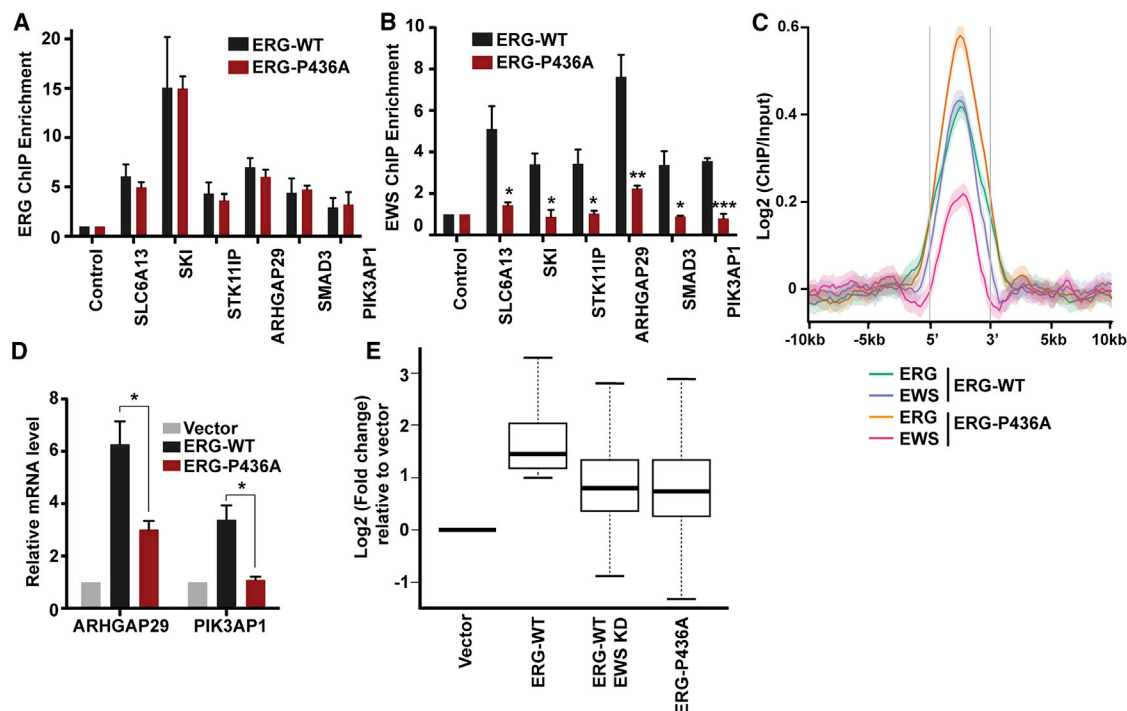


Figure 6. ERG Recruits EWS to Enhancers, Increasing Gene Expression

(A) Chromatin immunoprecipitation (ChIP) with anti-ERG antibody in RWPE1 cells expressing ERG or ERG-P436A. Enrichment indicates copies of indicated locus in the immunoprecipitate compared to a negative control locus (first two bars). Values are shown as mean and SEM (n = 3). Loci are labeled by the nearest gene. (B) ChIP as in (A), but with anti-EWS antibody.

(C) Ratio of ChIP-seq reads to input reads at 1,901 ERG-bound regions upon replicate ERG ChIP-seq or EWS ChIP-seq in RWPE1 cells expressing ERG or ERG-P436A.

(D) mRNA level of two ERG target genes by qRT-PCR in RWPE1 cells expressing the indicated construct shown relative to cells with empty vector. Values are shown as mean and SEM (n = 3).

(E) Box-plots show expression of 680 genes significantly activated by ERG in RWPE1 compared to vector only. Box shows median (25th and 75th percentiles), and bars encompass all values except outliers.

All p values (*p < 0.05, **p < 0.01, ***p < 0.001) were obtained by t test. See also Figure S6 and Table S2.

findings indicate that EWS acts as a co-activator for the majority of ERG target genes.

Oncogenic ETS and EWS Can Activate Transcription through Known Prostate Cancer and Ewing's Sarcoma *Cis*-Regulatory Sequences

We previously found that oncogenic ETS activate gene expression in prostate cells by binding *cis*-regulatory sequences consisting of neighboring ETS and AP-1 transcription factor binding sites, and these ETS/AP-1 sites occur near cell migration genes (Hollenhorst et al., 2011a). In Ewing's sarcoma, EWS-FLI1 activates transcription via *cis*-regulatory elements consisting of microsatellite repeats of the core ETS binding sequence, GGAA, and these GGAA repeats regulate the expression of genes that promote transformation and cancer cell survival (Gangwal et al., 2008; Riggi et al., 2014). However, GGAA repeats have not been identified as targets of oncogenic ETS factors in prostate cancer. Interestingly, an unbiased search of over-represented sequences in regions bound by both ERG and EWS in VCaP cells identified the GGAA repeat (Figure S1B). To expand this finding, datasets of ERG-, ETV1-, and ETV4-bound regions

in prostate cells were searched using the EWS-FLI1 position-weight matrix with a statistical boundary requiring at least four consecutive GGAA repeats (Table S3). Regions bound by ERG, ETV1, and ETV4 were significantly enriched for GGAA repeats compared to the random expectation (p < 0.0001 by χ^2). In contrast, non-ETS transcription factors such as MYC and JunD showed no enrichment for GGAA repeats (Table S3). To verify that EWS is present at ERG-bound GGAA repeat targets in VCaP cells, ChIP of ERG and EWS was assayed for enrichment of two GGAA-repeat-containing enhancers (*NEDD9* and *GALNT7*) and two ETS/AP-1-containing enhancers (*ARHGAP29* and *PIK3AP1*). All four enhancers showed enrichment of ERG and EWS compared to a negative control locus (Figure 7A). These data indicate that oncogenic ETS and EWS bind both ETS/AP-1 sequences and GGAA repeats in prostate cells.

To test if oncogenic ETS activate via both ETS/AP-1 sequences and GGAA repeats in prostate cells, two luciferase reporters were created, with one representing each type of enhancer. An ETS/AP-1-containing enhancer of the *FHL3* gene was cloned upstream of a minimal promoter controlling luciferase. Alternatively, seven consecutive copies of the sequence

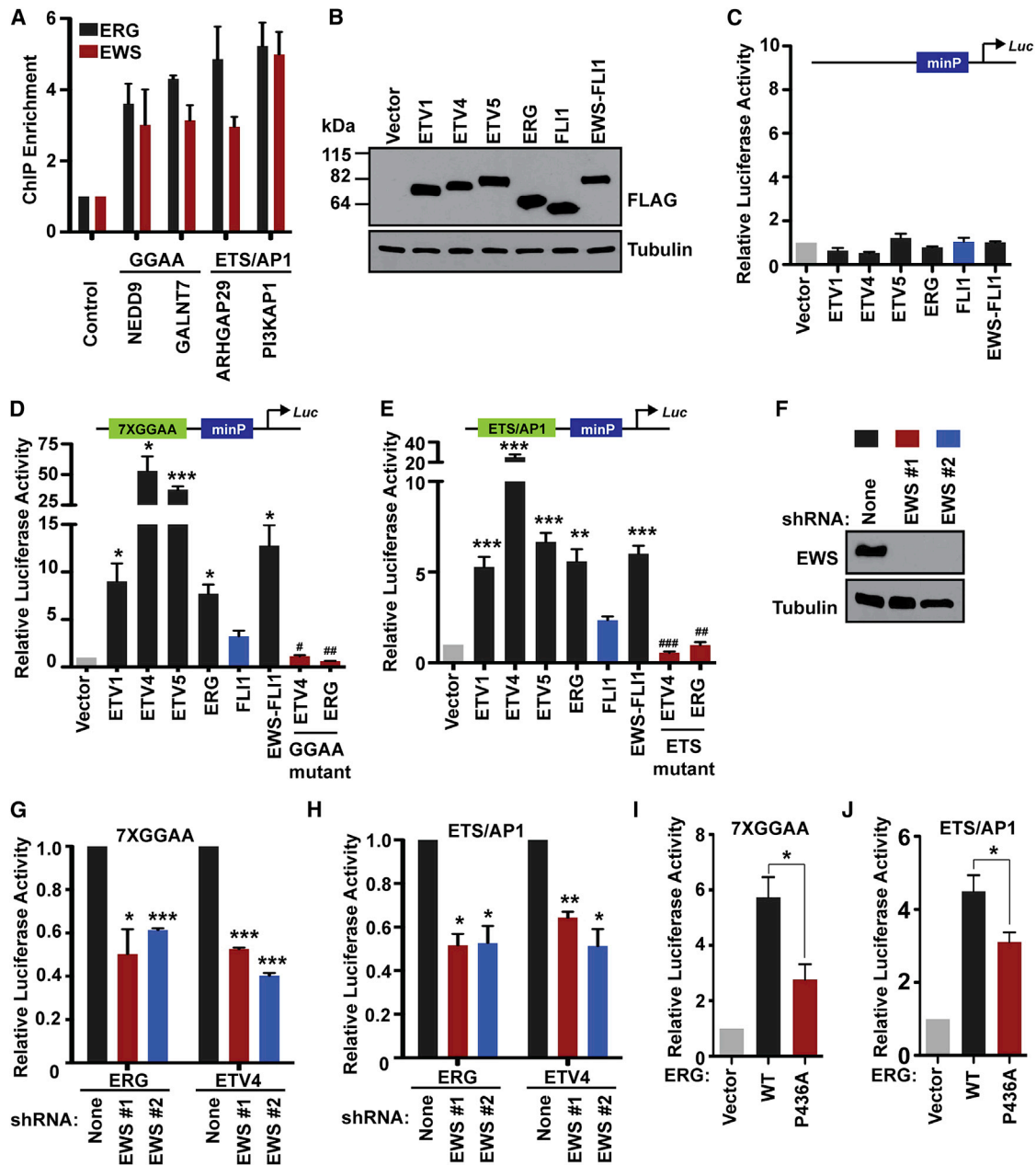


Figure 7. ERG and EWS Activate Transcription via Both ETS/AP-1 and GGAA Repeat Sequences

(A) ChIP enrichment using anti-ERG and anti-EWS antibodies in VCaP cells at two enhancers with GGAA repeats and two with ETS/AP-1 sequences compared to a control locus. Values are mean and SEM of three replicates.

(B) Immunoblot from RWPE1 cells transfected with the indicated FLAG-tagged constructs.

(C) Ratio of firefly to renilla (control) luciferase in RWPE1 cells transfected with minimal promoter firefly reporter and indicated expression construct, normalized to vector only. Values are mean and SEM (n = 3).

(D) As in (C), but with seven copies of GGAA prior to minimal promoter, except for last two columns which have seven copies of GAGA (GGAA mutant). p values (* < 0.05, ** < 0.01, *** < 0.001) by t test compare each ETS to FLI1, whereas p values denoted # compare mutant to wild-type reporter with the same ETS expressed.

(E) As in (D), except the reporter has a 474-bp region of an ETS/AP-1-containing enhancer present prior to the minimal promoter. The last two columns have the single ETS sequence mutated from GGAA to GAGA.

(F) EWS and tubulin immunoblots of RWPE1 cells stably expressing the indicated shRNAs.

(G) Relative luciferase as in (D) in RWPE1 cells expressing either ERG or ETV4 and shRNAs shown in (F).

(H) As in (G), but with the ETS/AP-1 reporter.

(I and J) Relative luciferase expression from 7xGGAA or the ETS/AP-1 reporter in RWPE1 cells expressing the indicated version of ERG shown relative to vector only. See also Table S3.

GGAA were cloned upstream of the minimal promoter. These two reporters, or the original minimal promoter vector, were then co-transfected into RWPE1 cells along with constructs expressing FLAG-tagged versions of ETV1, ETV4, ETV5, ERG, FLI1, and EWS-FLI1. Each of these proteins was expressed at similar levels (Figure 7B). Expression of these proteins had no effect on the empty vector containing the minimal promoter alone (Figure 7C), but activation was observed in the GGAA repeat reporter (Figure 7D) and the ETS/AP-1 reporter (Figure 7E). Mutation of each GGAA repeat to GAGA or mutation of the single ETS binding sequence in the ETS/AP-1 reporter eliminated activation by ETV4 and ERG (Figures 7D and 7E), indicating that an ETS binding sequence was required. Significantly, the four ETS proteins that interact with EWS caused significantly more activation of both reporters compared to FLI1, which does not interact with EWS (Figures 7D and 7E). Furthermore, the fusion of EWS to FLI1 (EWS-FLI1) activated the reporters significantly more than FLI1 and to a similar extent as ERG. These data indicate that both prostate cancer and Ewing's sarcoma ETS oncoproteins can activate through both ETS/AP-1 and GGAA repeat sequences.

We next tested if activation through ETS/AP-1 and GGAA-repeat sequences required EWS. First, ERG and ETV4 activation were tested in cell lines with EWS shRNA knockdowns (Figure 7F). Knockdown of EWS significantly decreased the activation of both ETS/AP-1 and GGAA-repeat reporters by both ERG and ETV4 (Figures 7G and 7H). Second, the function of the ERG P436A mutant, which does not interact with EWS, was assayed. Compared to wild-type ERG, ERG P436A had a decreased ability to activate both ETS/AP-1 and GGAA-repeat reporters (Figures 7I and 7J). Interestingly, EWS shRNA, ERG P436A, and FLI1 showed a similar ~50% lower activation of GGAA-repeat reporters compared to the ERG control (Figures 7D, 7G, and 7I), indicating that the difference between ERG and FLI1 transcriptional activation could be the ability of ERG to interact with EWS. Together, these data suggest that either through fusion (Ewing's sarcoma) or a protein-protein interaction (prostate cancer), EWS works with oncogenic ETS proteins to activate transcription of target genes via ETS/AP-1 and GGAA-repeat sequences.

DISCUSSION

These findings indicate that the common molecular mechanism of the ETS proteins that promote prostate cancer is an interaction with EWS. EWS binding to oncogenic ETS proteins was observed both in cells and using purified proteins, and this interaction was confined to four ETS family members: ERG, ETV1, ETV4, and ETV5. EWS was necessary for multiple oncogenic ETS functions, including transcriptional activation, cell migration, clonogenic growth, and transformation, but EWS was not necessary in cell lines lacking oncogenic ETS expression. A point mutant of ERG that failed to drive tumor growth was not defective in chromatin occupancy but lacked the ability to recruit EWS as a transcriptional co-activator. In summary, these findings reveal a highly specific protein-protein interaction necessary for oncogenic ETS function.

The interaction of EWS with ERG, ETV1, ETV4, and ETV5 is striking, because ERG shares no homology with ETV1, ETV4,

and ETV5 apart from the ETS DNA binding domain, which is present in all ETS proteins. Consistent with this, the regions of ERG and ETV5 that we mapped as the EWS interaction domains had no homology. This suggests that the EWS interaction with ERG may have evolved separately and may occur through a different interface compared to the interaction with ETV1, ETV4, and ETV5. It would also be expected that the EWS interaction is part of the normal biology of ERG, ETV1, ETV4, and ETV5 in the cell types where these proteins are normally expressed. In support of this idea, it has recently been shown that ETV1 and EWS cooperate to activate *FGF10* in developing limb buds and that both ETV1 and EWS bind the *FGF10* promoter (Yamamoto-Shiraishi et al., 2014).

Our data indicate that ERG has an oncogenic function that FLI1 lacks, because ERG interacts with EWS and FLI1 does not. However, gene rearrangements involving *FLI1* have been identified at low frequency in prostate tumors (Cancer Genome Atlas Research Network, 2015; Paulo et al., 2012). We offer two possible explanations for this discrepancy. First, it is possible that *FLI1* rearrangements are passenger mutations and not oncogenic. A recent study identified 3,106 different fusion transcripts in 150 metastatic prostate tumors (Robinson et al., 2015). Only one of these involved *FLI1*. There were 1,329 genes rearranged more often than *FLI1* in these tumors, and it is likely that many of these are passenger mutations. The second explanation is that *FLI1* is oncogenic but requires a mutational or signaling background not present in our system. For example, ERG requires PI3K/AKT activation to robustly promote tumorigenesis (Figure 5). It may be that *FLI1* requires activation of an unidentified signaling pathway to have the same function.

The role of EWS in oncogenic ETS function is likely to extend beyond roles in prostate cancer. In addition to an oncogenic function in prostate cancer, ERG is important for survival of leukemia cells (Tsuzuki et al., 2011), and ETV5 expression correlates with cell invasion in endometrial carcinoma (Planagumà et al., 2005). ETV4 has been reported to promote multiple tumor types, including colorectal, gastric, esophageal, ovarian, and non-small cell lung cancers (Oh et al., 2012). ETV1 plays important roles in melanoma, where 40% of tumors have copy gains of the *ETV1* gene (Jané-Valbuena et al., 2010). In gastrointestinal stromal tumors, the ETV1 protein is stabilized and cooperates with KIT mutations for cell transformation (Chi et al., 2010). Future work will be necessary to determine the importance of EWS interactions with ETS proteins in these tumor types.

EWS belongs to the FET family, which includes three highly conserved members: FUS, EWS, and TAF15 (Tan and Manley, 2009). Intriguingly, all three FET family proteins are fused to transcription factors in cancer. These include EWS-ETS fusions in Ewing's sarcoma and TLS-ERG fusion in myeloid leukemia (Arvand and Denny, 2001). In some cancers, the FET member in the fusion appears interchangeable, as in TLS-CHOP or EWS-CHOP fusions in myxoid liposarcoma, EWS-CHN or TAF15-CHN fusions in myxoid chondrosarcoma, and EWS-CIZ and TAF15-CIZ fusions in acute leukemia (Martini et al., 2002). In every fusion, the N-terminal domain of the FET protein is fused to a region of the transcription factor that includes the DNA binding domain. In the context of the fusion protein, the N-terminal domain from the FET protein has a strong transcriptional

activation function. These fusions do not alter DNA binding directly (May et al., 1993). However, a comparison of EWS-FLI1 and FLI1 genomic occupancy identified novel EWS-FLI1 bound enhancers (Patel et al., 2012). This is attributed to the ability of the EWS activation domain to alter local chromatin structure and create new enhancers (Patel et al., 2012; Riggi et al., 2014). Therefore it is interesting to postulate that the recruitment of EWS to enhancer regions by ERG would have a similar function and may be responsible for chromatin changes mediated by ERG (Elemento et al., 2012).

The wild-type EWS protein that interacts with ERG is multifunctional. EWS can interact with RNA polymerase II (RNAPII), heterogeneous nuclear ribonucleoproteins (A1, C1/C2, M and U), splicing factors (TASR-1 and YB-1), and RNA helicases (p68 and p72), suggesting roles in both transcription and in RNA processing (Bertolotti et al., 1998; Chansky et al., 2001; Pahlich et al., 2009; Yang et al., 1998, 2000). Accordingly, EWS can act as a co-activator for transcription factors such as OCT4, HNF4, YBX1, and ETV1 (Araya et al., 2003; Lee et al., 2005; Park et al., 2013; Yamamoto-Shiraishi et al., 2014) and as an alternative splicing factor (Paronetto et al., 2011, 2014). EWS-FLI1 has also been shown to alter splicing patterns (Selvanathan et al., 2015). It will be interesting to learn if the ETS factors that are rearranged in prostate cancer also have roles in RNA processing via EWS.

The ability of EWS to bind to, and function with, the ETS proteins that are expressed due to chromosomal rearrangements in prostate cancer indicates that this interaction is a key to oncogenic function. As such, EWS and the EWS-ETS interaction represent a highly specific target for prostate cancer treatment.

EXPERIMENTAL PROCEDURES

Protein Purification

ETS proteins were purified as described previously (Selvaraj et al., 2015). In brief, sequence verified constructs were cloned into pET28a (Novagen), expressed in *Escherichia coli* BL21 pRIL, and purified using Ni-NTA agarose resin (QIAGEN). pGEX-6p-2-GST-EWS (Pahlich et al., 2009) was obtained from Addgene (plasmid 46384). GST-EWS protein was expressed in BL21 pRIL, induced with IPTG (0.5 mM) at 22°C for 12–14 hr, and lysed by sonication in extraction buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 1% Nonidet P-40, 2 mM PMSF, 10% glycerol, and 30 mg lysozyme). Glutathione magnetic beads (Pierce) were added to the lysate, washed with GST wash buffer (125 mM Tris [pH 8.0] and 150 mM NaCl), and eluted (50 mM reduced glutathione in GST wash buffer).

Reporter Assay

The 474-bp (chromosome 1 [chr1]: 38465034–38465507, hg19) FHL3 enhancer was cloned into firefly luciferase reporter pGL4.25 (Promega). For the ETS site mutant, a sequence at position chr1: 38465405–38465402 was changed from GGAA to GAGA using standard methods. The GGAA repeat reporter was created by annealing cDNA oligonucleotides (described in Supplemental Experimental Procedures) containing seven tandem repeats of GGAA or GAGA and cloning into pGL4.25. A dual luciferase reporter assay (Promega) measured luciferase activity as described previously (Plotnik et al., 2014). Firefly luciferase values were normalized to renilla values.

RNA, Protein, and Affinity Purification from Cell Extracts

Antibodies for immunoblotting were ERG (CM 421, Biocare), EWS (sc-28327, Santa Cruz Biotechnology), ETV4 (ARP32263_P050, Aviva Systems Biology), pAKT (#4060, Cell Signaling Technology), tubulin (T9026, Sigma), and FLAG

(F1804, Sigma). Total protein extract from equal number of cells were separated on SDS-PAGE gels and immunoblotted using standard procedures. RNA levels were measured by reverse transcription followed by qRT-PCR with standard curves as described previously (Hollenhorst et al., 2011a). RNA levels were normalized to 18S rRNA. Purified His-tagged proteins were bound to Ni beads and incubated with PC3 cell extract. For more information and oligo sequences, see Supplemental Experimental Procedures.

ChIP, ChIP-Seq, and RNA Seq

ChIP of indicated proteins was performed from VCaP and RWPE1 cells as described previously (Hollenhorst et al., 2011a) using the same antibodies described for immunoblotting, with slight modifications. Total RNA from three biological replicates of RWPE1 cells expressing vector, ERG-WT, ERG-P436A, or ERG-WT with EWS knockdown shRNA was isolated and sequenced independently. For further information, see Supplemental Experimental Procedures.

Viral Transductions and Cell Line Assays

VCaP and PC3 cell lines were authenticated within 6 months of use by a PowerPlex 16HS assay (Promega). RWPE1 and RWPE-KRAS (RWPE2) lines were obtained from ATCC and passaged less than 25 times. All lines were cultured according to ATCC guidelines. Retroviral vectors stably overexpressed proteins and lentiviral vectors provided stable shRNA expression. Migration and anchorage-independent growth assays were performed as described previously (Hollenhorst et al., 2011c), with modifications. For more information, see Supplemental Experimental Procedures.

In Vivo Xenograft Tumor Growth Model

All animal protocols described in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the Indiana University School of Medicine. 2×10^6 RWPE1 epithelial cells expressing ERG, activated Akt (Myr-Akt), both ERG and Akt (ERG/Myr-Akt), mutant ERG with Akt (P436A/Myr-Akt), or vector control (all as described above) were combined with an equal volume of Matrigel and 0.5×10^6 cancer-associated fibroblast cells and injected subcutaneously on the flanks of nude mice. Tumors were measured weekly with calipers and tumor volume was calculated as the volume of a spheroid using the formula: volume = length \times width \times height \times 0.5236. Controls included each epithelial cell line without fibroblasts and C4-2 prostate cancer cells with cancer associated fibroblasts (CAFs). Upon completion of the experiment after 8 weeks, all tumors were harvested, verified as tumor, and processed for histology (10% buffered formalin, processing through a gradient of ethanol and xylene and embedding in paraffin, and then sectioning and H&E staining).

Statistical Analysis

Student's t test was applied to data with at least three independent biological replicates to calculate significance. A χ^2 test with Yates correction was applied for ChIP-seq analysis. Benjamini-Hochberg correction was used to calculate adjusted p value for differential gene expression analysis.

ACCESSION NUMBERS

The accession numbers for the ChIP-seq and RNA-sequencing data used in this study are GEO: GSE73616 and GEO: GSE81493, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.10.001>.

AUTHOR CONTRIBUTIONS

V.K., N.S., T.R.N., J.P.P., and T.J.J. designed and performed the experiments and, together with P.C.H., analyzed the data. V.K. and P.C.H. wrote the manuscript with input from all authors.

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REFERENCES

- Araya, N., Hirota, K., Shimamoto, Y., Miyagishi, M., Yoshida, E., Ishida, J., Kaneko, S., Kaneko, M., Nakajima, T., and Fukamizu, A. (2003). Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. *J. Biol. Chem.* 278, 5427–5432.
- Arvand, A., and Denny, C.T. (2001). Biology of EWS/ETS fusions in Ewing's family tumors. *Oncogene* 20, 5747–5754.
- Aytes, A., Mitrofanova, A., Kinkade, C.W., Lefebvre, C., Lei, M., Phelan, V., Lekay, H.C., Koutcher, J.A., Cardiff, R.D., Califano, A., et al. (2013). ETV4 promotes metastasis in response to activation of PI3-kinase and Ras signaling in a mouse model of advanced prostate cancer. *Proc. Natl. Acad. Sci. U S A* 110, E3506–E3515.
- Baena, E., Shao, Z., Linn, D.E., Glass, K., Hamblen, M.J., Fujiwara, Y., Kim, J., Nguyen, M., Zhang, X., Godinho, F.J., et al. (2013). ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. *Genes Dev.* 27, 683–698.
- Bertolotti, A., Melot, T., Acker, J., Vigneron, M., Delattre, O., and Tora, L. (1998). EWS, but not EWS-FLI-1, is associated with both TFIID and RNA polymerase II: interactions between two members of the TET family, EWS and hTAFII68, and subunits of TFIID and RNA polymerase II complexes. *Mol. Cell. Biol.* 18, 1489–1497.
- Cancer Genome Atlas Research Network (2015). The molecular taxonomy of primary prostate cancer. *Cell* 163, 1011–1025.
- Carver, B.S., Tran, J., Gopalan, A., Chen, Z., Shaikh, S., Carracedo, A., Ailamonti, A., Nardella, C., Varmeh, S., Scardino, P.T., et al. (2009). Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nat. Genet.* 41, 619–624.
- Chansky, H.A., Hu, M., Hickstein, D.D., and Yang, L. (2001). Oncogenic TLS/ERG and EWS/Flt-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. *Cancer Res.* 61, 3586–3590.
- Chi, P., Chen, Y., Zhang, L., Guo, X., Wongvipat, J., Shamu, T., Fletcher, J.A., Dewell, S., Maki, R.G., Zheng, D., et al. (2010). ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature* 467, 849–853.
- Chng, K.R., Chang, C.W., Tan, S.K., Yang, C., Hong, S.Z., Sng, N.Y., and Cheung, E. (2012). A transcriptional repressor co-regulatory network governing androgen response in prostate cancers. *EMBO J.* 31, 2810–2823.
- Clark, J., Merson, S., Jhavar, S., Flohr, P., Edwards, S., Foster, C.S., Eeles, R., Martin, F.L., Phillips, D.H., Crundwell, M., et al. (2007). Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. *Oncogene* 26, 2667–2673.
- Delattre, O., Zucman, J., Plougastel, B., Desmaziere, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., et al. (1992). Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* 359, 162–165.
- Elemento, O., Rubin, M.A., and Rickman, D.S. (2012). Oncogenic transcription factors as master regulators of chromatin topology: a new role for ERG in prostate cancer. *Cell Cycle* 11, 3380–3383.
- Gangwal, K., Sankar, S., Hollenhorst, P.C., Kinsey, M., Haroldsen, S.C., Shah, A.A., Boucher, K.M., Watkins, W.S., Jorde, L.B., Graves, B.J., and Lessnick, S.L. (2008). Microsatellites as EWS/FLI response elements in Ewing's sarcoma. *Proc. Natl. Acad. Sci. USA* 105, 10149–10154.
- Hollenhorst, P.C., Jones, D.A., and Graves, B.J. (2004). Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res.* 32, 5693–5702.
- Hollenhorst, P.C., Ferris, M.W., Hull, M.A., Chae, H., Kim, S., and Graves, B.J. (2011a). Oncogenic ETS proteins mimic activated RAS/MAPK signaling in prostate cells. *Genes Dev.* 25, 2147–2157.
- Hollenhorst, P.C., McIntosh, L.P., and Graves, B.J. (2011b). Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu. Rev. Biochem.* 80, 437–471.
- Hollenhorst, P.C., Paul, L., Ferris, M.W., and Graves, B.J. (2011c). The ETS gene ETV4 is required for anchorage-independent growth and a cell proliferation gene expression program in PC3 prostate cells. *Genes Cancer* 1, 1044–1052.
- Jané-Valbuena, J., Widlund, H.R., Perner, S., Johnson, L.A., Dibner, A.C., Lin, W.M., Baker, A.C., Nazarian, R.M., Vijayendran, K.G., Sellers, W.R., et al. (2010). An oncogenic role for ETV1 in melanoma. *Cancer Res.* 70, 2075–2084.
- King, J.C., Xu, J., Wongvipat, J., Hieronymus, H., Carver, B.S., Leung, D.H., Taylor, B.S., Sander, C., Cardiff, R.D., Couto, S.S., et al. (2009). Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. *Nat. Genet.* 41, 524–526.
- Lee, J., Rhee, B.K., Bae, G.Y., Han, Y.M., and Kim, J. (2005). Stimulation of Oct-4 activity by Ewing's sarcoma protein. *Stem Cells* 23, 738–751.
- Martini, A., La Starza, R., Janssen, H., Bilhou-Nabera, C., Corveleyn, A., Somers, R., Aventin, A., Foà, R., Hagemeijer, A., Mecucci, C., and Marynen, P. (2002). Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. *Cancer Res.* 62, 5408–5412.
- May, W.A., Lessnick, S.L., Braun, B.S., Klemsz, M., Lewis, B.C., Lunsford, L.B., Hromas, R., and Denny, C.T. (1993). The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. *Mol. Cell. Biol.* 13, 7393–7398.
- Oh, S., Shin, S., and Janknecht, R. (2012). ETV1, 4 and 5: an oncogenic subfamily of ETS transcription factors. *Biochim. Biophys. Acta* 1826, 1–12.
- Pahlich, S., Quero, L., Roschitzki, B., Leemann-Zakaryan, R.P., and Gehring, H. (2009). Analysis of Ewing sarcoma (EWS)-binding proteins: interaction with hnRNP M, U, and RNA-helicases p68/72 within protein-RNA complexes. *J. Proteome Res.* 8, 4455–4465.
- Park, J.H., Kang, H.J., Kang, S.I., Lee, J.E., Hur, J., Ge, K., Mueller, E., Li, H., Lee, B.C., and Lee, S.B. (2013). A multifunctional protein, EWS, is essential for early brown fat lineage determination. *Dev. Cell* 26, 393–404.
- Paronetto, M.P., Miñana, B., and Valcárcel, J. (2011). The Ewing sarcoma protein regulates DNA damage-induced alternative splicing. *Mol. Cell* 43, 353–368.
- Paronetto, M.P., Bernardis, I., Volpe, E., Bechara, E., Sebestyén, E., Eyra, E., and Valcárcel, J. (2014). Regulation of FAS exon definition and apoptosis by the Ewing sarcoma protein. *Cell Rep.* 7, 1211–1226.
- Patel, M., Simon, J.M., Iglesia, M.D., Wu, S.B., McFadden, A.W., Lieb, J.D., and Davis, I.J. (2012). Tumor-specific retargeting of an oncogenic transcription factor chimera results in dysregulation of chromatin and transcription. *Genome Res.* 22, 259–270.
- Paulo, P., Barros-Silva, J.D., Ribeiro, F.R., Ramalho-Carvalho, J., Jerónimo, C., Henrique, R., Lind, G.E., Skotheim, R.I., Lothe, R.A., and Teixeira, M.R. (2012). FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. *Genes Chromosomes Cancer* 51, 240–249.
- Planagumà, J., Abal, M., Gil-Moreno, A., Díaz-Fuertes, M., Monge, M., García, A., Baró, T., Xercavins, J., Reventós, J., and Alameda, F. (2005). Up-regulation of ERM/ETV5 correlates with the degree of myometrial infiltration in endometrioid endometrial carcinoma. *J. Pathol.* 207, 422–429.

- Plotnik, J.P., Budka, J.A., Ferris, M.W., and Hollenhorst, P.C. (2014). ETS1 is a genome-wide effector of RAS/ERK signaling in epithelial cells. *Nucleic Acids Res.* 42, 11928–11940.
- Rickman, D.S., Pflueger, D., Moss, B., VanDoren, V.E., Chen, C.X., de la Taille, A., Kuefer, R., Tewari, A.K., Setlur, S.R., Demichelis, F., and Rubin, M.A. (2009). SLC45A3-ELK4 is a novel and frequent erythroblast transformation-specific fusion transcript in prostate cancer. *Cancer Res.* 69, 2734–2738.
- Riggi, N., Knoechel, B., Gillespie, S.M., Rheinbay, E., Boulay, G., Suvà, M.L., Rossetti, N.E., Boonseng, W.E., Oksuz, O., Cook, E.B., et al. (2014). EWS-FLI1 utilizes divergent chromatin remodeling mechanisms to directly activate or repress enhancer elements in Ewing sarcoma. *Cancer Cell* 26, 668–681.
- Robinson, D., Van Allen, E.M., Wu, Y.M., Schultz, N., Lonigro, R.J., Mosquera, J.M., Montgomery, B., Taplin, M.E., Pritchard, C.C., Attard, G., et al. (2015). Integrative clinical genomics of advanced prostate cancer. *Cell* 161, 1215–1228.
- Selvanathan, S.P., Graham, G.T., Erkizan, H.V., Dirksen, U., Natarajan, T.G., Dakic, A., Yu, S., Liu, X., Paulsen, M.T., Ljungman, M.E., et al. (2015). Oncogenic fusion protein EWS-FLI1 is a network hub that regulates alternative splicing. *Proc. Natl. Acad. Sci. USA* 112, E1307–E1316.
- Selvaraj, N., Budka, J.A., Ferris, M.W., Jerde, T.J., and Hollenhorst, P.C. (2014). Prostate cancer ETS rearrangements switch a cell migration gene expression program from RAS/ERK to PI3K/AKT regulation. *Mol. Cancer* 13, 61.
- Selvaraj, N., Kedage, V., and Hollenhorst, P.C. (2015). Comparison of MAPK specificity across the ETS transcription factor family identifies a high-affinity ERK interaction required for ERG function in prostate cells. *Cell Commun. Signal.* 13, 12.
- Shaw, A., Gipp, J., and Bushman, W. (2010). Exploration of Shh and BMP paracrine signaling in a prostate cancer xenograft. *Differentiation* 79, 41–47.
- Svensson, M.A., Lafargue, C.J., Macdonald, T.Y., Pflueger, D., Kitabayashi, N., Santa-Cruz, A.M., Garsha, K.E., Sathyanarayana, U.G., Riley, J.P., Yun, C.S., et al. (2011). Testing mutual exclusivity of ETS rearranged prostate cancer. *Lab. Invest.* 91, 404–412.
- Tan, A.Y., and Manley, J.L. (2009). The TET family of proteins: functions and roles in disease. *J. Mol. Cell Biol.* 1, 82–92.
- Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., et al. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310, 644–648.
- Tomlins, S.A., Laxman, B., Dhanasekaran, S.M., Helgeson, B.E., Cao, X., Morris, D.S., Menon, A., Jing, X., Cao, Q., Han, B., et al. (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* 448, 595–599.
- Tomlins, S.A., Laxman, B., Varambally, S., Cao, X., Yu, J., Helgeson, B.E., Cao, Q., Prensner, J.R., Rubin, M.A., Shah, R.B., et al. (2008). Role of the TMPRSS2-ERG gene fusion in prostate cancer. *Neoplasia* 10, 177–188.
- Tsuzuki, S., Taguchi, O., and Seto, M. (2011). Promotion and maintenance of leukemia by ERG. *Blood* 117, 3858–3868.
- Wang, X.S., Shankar, S., Dhanasekaran, S.M., Ateeq, B., Sasaki, A.T., Jing, X., Robinson, D., Cao, Q., Prensner, J.R., Yocum, A.K., et al. (2011). Characterization of KRAS rearrangements in metastatic prostate cancer. *Cancer Discov.* 1, 35–43.
- Wu, L., Zhao, J.C., Kim, J., Jin, H.J., Wang, C.Y., and Yu, J. (2013). ERG is a critical regulator of Wnt/LEF1 signaling in prostate cancer. *Cancer Res.* 73, 6068–6079.
- Yamamoto-Shiraishi, Y., Higuchi, H., Yamamoto, S., Hirano, M., and Kuroiwa, A. (2014). ETV1 and EWSR1 cooperatively regulate limb mesenchymal Fgf10 expression in response to apical ectodermal ridge-derived fibroblast growth factor signal. *Dev. Biol.* 394, 181–190.
- Yang, L., Embree, L.J., Tsai, S., and Hickstein, D.D. (1998). Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing. *J. Biol. Chem.* 273, 27761–27764.
- Yang, L., Chansky, H.A., and Hickstein, D.D. (2000). EWS.Fli-1 fusion protein interacts with hyperphosphorylated RNA polymerase II and interferes with serine-arginine protein-mediated RNA splicing. *J. Biol. Chem.* 275, 37612–37618.
- Yu, J., Yu, J., Mani, R.S., Cao, Q., Brenner, C.J., Cao, X., Wang, X., Wu, L., Li, J., Hu, M., et al. (2010). An integrated network of androgen receptor, polycarb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell* 17, 443–454.
- Zong, Y., Xin, L., Goldstein, A.S., Lawson, D.A., Teitell, M.A., and Witte, O.N. (2009). ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. *Proc. Natl. Acad. Sci. USA* 106, 12465–12470.